

## Biotinylated Micro-RNA Pull Down Assay for Identifying miRNA Targets

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**[Abstract]** microRNA (miRNA) directly associates with its target transcripts (mRNA). This protocol describes a method for detection of direct interaction between miRNA and mRNA. The result of interaction helps screening the specific target mRNAs for a miRNA.

**Keywords:** miRNA, Biotin-pulldown, miRNA-mRNA interaction, Transfection, Biotin labelling

**[Background]** MiRNAs are small non coding regulatory RNAs. MiRNAs typically control the gene expression by binding to complementary sequence in their target mRNAs. Many bioinformatics and computational programs are available to predict miRNA targets. But experimental methods identifying direct association of miRNA with target mRNA are very limited. The current protocol can be very helpful in identifying miRNA targets (Phatak *et al.*, 2016).

### Materials and Reagents

1. 10 cm tissue culture dishes
2. 1.5 ml microfuge tube
3. PCR tubes
4. 15 and 50 ml polypropylene tubes
5. Q-PCR plates
6. Cells
7. Custom synthesis of miRNA sequence with 3'BIOTIN manipulation
8. Phosphate buffered saline (PBS)
9. Protease inhibitor (100x cocktail) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 78430)
10. RNase inhibitor (Thermo Fisher Scientific, catalog number: EO0381)
11. Streptavidin-Dyna beads (Dyna beads M-280 streptavidin) (Thermo Fisher Scientific, Invitrogen™, catalog number: 11205D)
12. Yeast tRNA (stock 10 mg/ml) (Thermo Fisher Scientific, Ambion™, catalog number: AM7119)
13. TRIzol (Thermo Fisher Scientific, Ambion™, catalog number: 10296028)
14. Chloroform (Sigma-Aldrich, catalog number: C2432)
15. 2-propanol (Sigma-Aldrich, catalog number: I9516)
16. GlycoBlue™ coprecipitant (Thermo Fisher Scientific, Invitrogen™, catalog number: AM9515)

17. 200% proof ethanol (Sigma-Aldrich, catalog number: E7023)
18. Nuclease free water (Quality Biological, catalog number: 351-029-721)
19. Reverse transcription system (Promega, catalog number: A3500)
20. Micro RNA RT Kit (Thermo Fisher Scientific, Applied Biosystem™)
21. miRNeasy Mini Kit (QIAGEN, catalog number: 217004)
22. Qiashredder (QIAGEN, catalog number: 79656)
23. RNAiMax (Thermo Fisher Scientific, Invitrogen™, catalog number: 13778150)
24. Potassium chloride (KCl)
25. Magnesium chloride (MgCl<sub>2</sub>)
26. Tris-hydrochloride (pH 7.5)
27. IGEPAL CA-630 (Sigma-Aldrich, catalog number: I8896)
28. Sodium hydroxide (NaOH)
29. Sodium chloride (NaCl)
30. Lysis buffer (see Recipes)
31. Solution A (see Recipes)
32. Solution B (see Recipes)

### **Equipment**

1. Magnetic separation Stand Magnesphere® technology (Promega, catalog number: Z5342)
2. Mini tube rotator (Fisher Scientific, model: Fisher Scientific™ Mini-Tube rotators, catalog number: 05-450-127)
3. Refrigerated microfuge Legend™ micro 21R (Thermo Fisher Scientific, model: Sorvall™ Legend™ 21R)
4. ABI Step one plus Q-PCR system (Thermo Fisher Scientific, Applied Biosystem™, model: StepOnePlus™ Real-Time PCR System, catalog number: 4376599)
5. Veriti 96 well Thermal Cycler 0.2 ml alloy (Thermo Fisher Scientific, Applied Biosystem™, model: Veriti® 96-Well Thermal Cycler, catalog number: 4375786)
6. -20 °C freezer
7. Vortexer

### **Procedure**

miRNA of interest labeled with biotin at 3' end and a scrambled control miRNA were commercially synthesized (Phatak *et al.*, 2016) from Dharmacon. Seed the cells one day before transfection in 10 cm tissue culture dish in duplicate. Cells should be seeded at a density so that they are not > 80% confluent at the time of collection. Twenty four hours later, transfect the cells with control miRNA and 3' biotin-labeled miRNA at a final concentration of 10-100 nM. RNAimax was used as a transfection reagent. Transfection was done according to manufacturer's guideline. Forty eight hours post transfection, whole

cell lysates were harvested. (Time and concentration needs an optimization since it varies with cell line and miRNA). Medium may be changed one day after transfection.

#### A. Cell lysate preparation

1. Remove medium from dish and wash the cells carefully twice with ice-cold 1x PBS. Scrape or trypsinize cells, spin at 2,000  $\times g$  for 5 min at 4 °C. Pre-chill microcentrifuge tubes before collecting pellet.

*Note: Pellet can be stored at -80 °C at this point.*

2. If continuing with lysates then add 550  $\mu$ l lysis buffer supplemented with protease inhibitor (PI) and RNase inhibitor. (Always add PI and RNase inhibitor at the time of lysis). Mix cells with lysis buffer by pipetting up and down couple of times. Incubate on ice for 10 min. Centrifuge the cell lysates at 4 °C, 18,000  $\times g$  for 10 min. Collect the supernatant into a new microcentrifuge tube.
3. Simultaneously, prepare Streptavidin-Dyna beads (50  $\mu$ l for each sample).
4. Wash the beads twice with 500  $\mu$ l solution A.
5. Wash the beads twice with 500  $\mu$ l solution B.
6. Wash the beads three times with 1 ml lysis buffer. During every wash put the tube on a magnetic stand and remove the supernatant carefully without taking the beads out.
7. Incubate the beads with 500  $\mu$ l lysis buffer and 10  $\mu$ l yeast tRNA on a rotator at 4 °C for 2 h.

#### B. Pull-down

1. After 2 h of incubation, put the tube containing the beads on the magnetic stand, take out the supernatant and wash the beads once with 1 ml lysis buffer.
2. Mix 500  $\mu$ l lysates with the beads and incubate at 4 °C on the rotator overnight. Save left over 50  $\mu$ l lysate at -80 °C, which would be used as input.
3. Next day, centrifuge the tube briefly and put it on magnetic stand and remove the supernatant. Wash the beads 4 times with 1 ml lysis buffer.

#### C. RNA isolation

1. Take out the saved lysates from -80 °C, thaw it on ice.
2. Add 750  $\mu$ l Trizol LS per sample and 250  $\mu$ l water to the input and the pull down beads and mix well. Keep the tubes at -20 °C freezer for minimum 2 h. This is additional stopping point. The tubes can be kept at -20 °C for overnight.
3. Thaw the mixture at room temperature, add 200  $\mu$ l chloroform, vortex for 45 sec and keep at room temperature for 2-3 min.
4. Centrifuge at 4 °C 18,000  $\times g$  for 15 min.  
*Note: Turn on centrifuge in advance so that by the time the tubes are ready to spin it is already at 4 °C.*
5. Transfer the upper layer to new tubes (it will be ~500-600  $\mu$ l). Discard the old tubes.
6. Add equal volume of 2-propanol and 5  $\mu$ l glycoblue, mix by inverting the tubes a few times. Incubate at RT for 10 min.

7. Centrifuge at 4 °C 18,000 x g for 15 min. Discard the supernatant very carefully. Wash the pellet twice with 1 ml prechilled 70% ethanol.
8. Carefully remove the supernatant while washing. Air dry the pellet and resuspend it in 20 µl nuclease free water.

#### D. RT-PCR

1. Use whole 20 µl RNA from biotin-labeled sample and 1 µg RNA from the input sample for mRNA RT reactions. Set up the mRNA RT reactions according to manufacturer's instructions.
2. Use 10 ng of total RNA per 15 µl reactions using TaqMan miRNA Reverse Transcription Kit with specific miRNA primer for the input samples. This is required to measure miRNA levels.
3. Run q-PCR in triplicate for target mRNAs with cDNA made by mRNA-RT Kit. Volume of cDNA will need an optimization as binding capacity would vary for target. If possible please include a positive control (known target for particular miRNA). GAPDH can be used as loading control.
4. For micro RNA levels, run separate q-PCR using micro-RNA cDNA in triplicate. Small nuclear RNA U6 can be used for loading control.

#### Data analysis

Calculate the miRNA enrichment as follows:

Bi-miR-214-3p pull-down for CUGBP1 mRNA/Scramble control pull-down for CUGBP1mRNA = X

Bi-miR-214-3p input/Scramble control input = Y (saved in step 2 under pull down paragraph)

Fold binding = X/Y.

Results are expressed as the means ± SD from three independent experiments with minimum three replicates for each set of experiment (Phatak *et al.*, 2016). Data derived from multiple determinations were subjected to two-tailed Student's *t*-test and *P* values < 0.05 were considered statistically significant.

#### Recipes

1. Lysis buffer
  - 20 mM Tris-HCl (pH 7.5)
  - 100 mM KCl
  - 5 mM MgCl<sub>2</sub>
  - 0.3% IGEPAL CA-630
2. Solution A
  - 0.1 M NaOH
  - 0.05 M NaCl
3. Solution B
  - 0.1 M NaCl

70% ethanol

Dilute with nuclease free water from 200 proof 100% ethanol and prechilled before using

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Conflicts of interest: None.

This protocol was adapted and modified as described previously (Orom and Lund, 2007; Choi *et al.*, 2014)

### **References**

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